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Recommended Citation

Gonzalez E, Bui M, Ahmed AA. IGF1R immunohistochemistry in Ewing's sarcoma as predictor of response to targeted therapy. *Int J Health Sci (Qassim)*. 2020;14(4):17-21.

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IGF1R immunohistochemistry in Ewing's sarcoma as predictor of response to targeted therapy

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ISSN: 1658-3639

PUBLISHER: Qassim University

Introduction

Ewing's sarcoma tumor (EST) is the second most common sarcoma of bone in children and adults that is associated with metastatic tendency in one-third of cases and subsequent high mortality.^[1,2] Although patients with localized disease can be cured, metastatic tumors are resistant to conventional therapy prompting the need to identify additional venues for biology-based treatment.^[1,2]

Upregulation and stimulation of insulin growth factor (IGF) receptors in EST play important roles in tumor growth and maintenance. The beta subunit of the IGF1 receptor (IGF1R β) spans the cell membrane and is the main subunit responsible for IGF signal transduction, which subsequently activates mitogen-activated protein and extracellular signal-regulated protein kinases (MAPK), phosphoinositide 3-kinases, and other proliferative pathways.^[3,4] S6 kinase (S6K) is a family of cytoplasmic ribosomal

ABSTRACT

Objectives: Ewing's sarcoma is an aggressive malignancy of bone and soft tissue in children and young adults. Despite advances in modern therapy, metastasis can occur and results in high mortality. The objective of this study was to identify whether the signaling transduction proteins, insulin growth factor receptor (IGF1R) and S6 kinase (S6K), can predict poor prognosis in Ewing's sarcoma.

Methods: After the Institutional Research Board approval, immunohistochemical experiments on tissue microarray slides containing 32 archived Ewing's sarcoma tumor samples were performed with antibodies against IGF1R β and p-S6K. Immunohistochemical staining results were correlated with patients' clinical data including clinical stage and overall survival (OS).

Results: Patients had an age range of 12–72 years and 8 (25%) were ≤ 20 years. After a follow-up to 14 years, the OS ranged from 25 to 5065 days. High expression of IGF1R β and p-S6K, defined as staining stronger than positive control, was identified in 25% and 68.75% of cases, respectively. Statistical analysis revealed that IGF1R β high expression had a significant association with adverse outcome, shorter OS ($P < 0.05$), and near significant association with advanced stage tumors ($P = 0.0534$). Expression of S6K exhibited a trend toward shorter survival ($P = 0.0934$).

Conclusion: High expression or strong staining of IGF1R β in Ewing's sarcoma may be more important than overall positive staining in identifying poor prognosis and aggressive cases to be selected for IGF1R inhibitory therapy. More definitive studies are needed to confirm the role of S6K in the prognosis in Ewing's sarcoma tumors.

Keywords: Ewing's sarcoma, insulin growth factor 1R β , immunohistochemistry, S6 kinase, tissue microarray

protein kinases that act to promote cell survival and growth. It activates the 40S ribosomal subunits involved in regulating translation and inactivates apoptosis through phosphorylation of the proapoptotic molecule Bcl-2-associated death promoter BAD.^[5] Phosphorylated S6K becomes activated by MAPK and mammalian target of rapamycin pathways and hence relays signaling from growth factor receptors to the nucleus [Figure 1].

The consistent expression of IGF1R in pre-clinical experiments and tumor regression with IGF1R inhibition has led to the development of several clinical trials IGF1R inhibitors.^[6] Most of these trials have reported variable clinical response with complete response in $< 20\%$ of cases.^[6] At present, there are no validated methods to predict such good responders. In this study, we evaluate the expression of IGF1R β in EST, based on the hypothesis that its high expression in clinical specimens may serve as indicator of potential clinical response to targeted therapy.

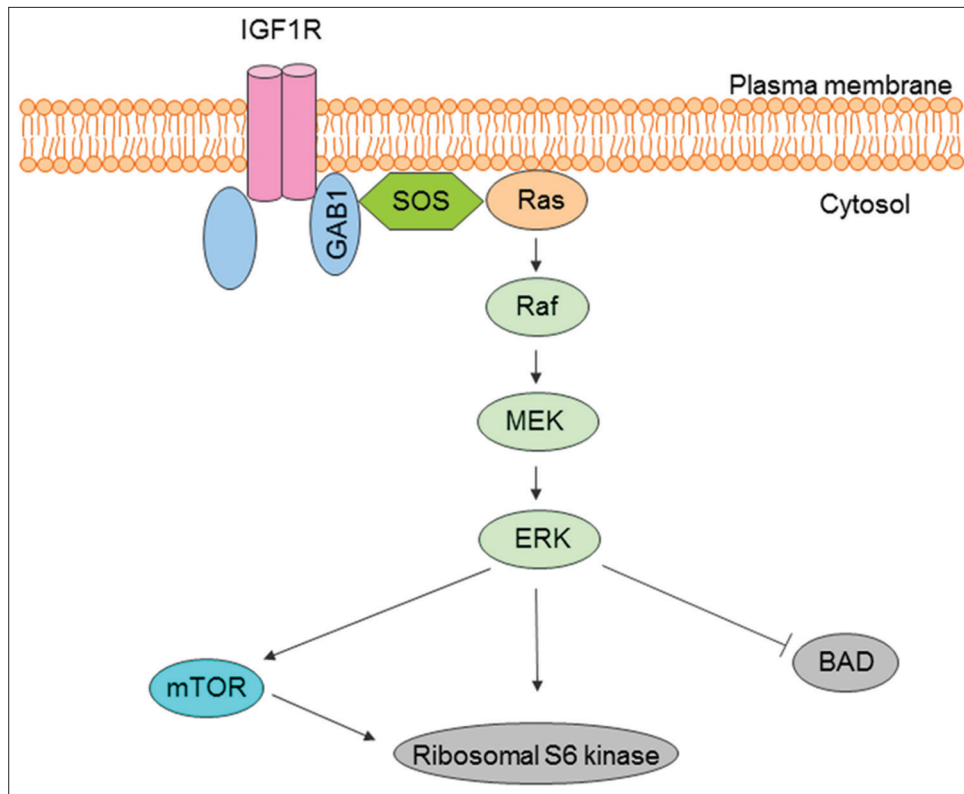


Figure 1: Schematic representation of the insulin growth factor pathway and its relationship to intracellular signaling molecules

Materials and Methods

Patients and specimens

After an Institutional Review Board approval, tissue microarray (TMA) slides were collected from patients with primary and metastatic EST over a period of 13 years (1995–2007). Patient consents were waived because of archival nature of the study. Initial diagnostic biopsies and resection specimens were collected from the archives of pathology department at Moffitt Cancer Center, after confirming the diagnosis by immunohistochemistry (IHC) and molecular methods. Exclusion criteria include insufficient tumor volume for the microarray. Patient's age, sex, clinical stage, and follow-up data were also compiled as well as follow-up information up to 14 years after diagnosis. The overall survival (OS) was calculated (in days) from initial diagnosis until death or last known follow-up. After confirming the diagnosis of Ewing's sarcoma, representative paraffin-embedded tumor cores (0.6 mm in diameter) from initial diagnostic and pre-treatment specimens were compiled for the construction of TMA blocks according to a previously described method.^[7] Two cores were obtained from each tumor and placed adjacently in the TMA blocks.

IHC

Sections for IHC were stained with antibodies for the signaling proteins, IGF1R β (Leica Biosystems, Buffalo Grove, IL)

and p-S6K (Cell Signaling, Danvers, MA) according to the manufacturer's specifications. Automated immunostaining was performed with a Leica Bond Max instrument (Leica, Richmond, Illinois). After deparaffinization and antigen retrieval, tumor microarray and control slides were sequentially incubated with the primary antibody, a secondary antibody, a polymer conjugate, and a coloring reagent that gives a brown color staining. Separate positive controls were made from breast carcinoma, all of which were previously shown to exhibit expression of the studied proteins. Negative controls were stained similarly except for omission of primary antibodies. Staining of vascular endothelial cells served as internal positive control.

Analysis of results

IHC staining results were recorded by two pathologists. Identifiable staining was classified as low (weak or less than that of internal positive control) or high (equal or stronger than internal positive control). Because of the limited specimen sizes, data on the extent of the staining (focal versus diffuse) were not collected. For statistical purposes, negative and low staining results were grouped together and compared to high staining (high expression). Kaplan–Meier survival curves were also plotted to demonstrate any difference in survival between the staining groups of the selected proteins. The association of other clinicopathologic parameters and IHC expression was also examined by Fisher exact Chi-square test using an online software <http://www>.

socscistatistics.com/tests/chisquare. $P < 0.05$ was considered statistically significant.

Results

Patients and tumors

Clinical and survival data were collected from an initial cohort of 36 patients whose clinical information was previously published.^[8] Subsequent review of the stained sections revealed loss of stainable tissue in four cases. Of the remaining 32 patients, 21 were male and 11 females (M:F=1.9) and had an age range of 12–72 years. Twenty-four (75%) patients were older than 20 years and 8 (25%) were pediatric (≤ 20 years). Patients had follow-up visits extending from 25 days to 14 years from the primary diagnosis. The OS ranged from 25 to 5065 days.

Tumor samples were from primary locations in 26 patients and from metastatic sites in six patients. Of the primary locations, 18 (56.25%) patients had skeletal tumors and 8 (25%) tumors were extraskeletal. Tumor sizes ranged from 1.2 to 34.5 cm (measured in 22 patients). Clinical stage was determined in 28 cases and was grouped into localized (12 cases), regional extension (8 cases), and distant metastasis (8 cases), Table 1.

IHC results and statistical analysis

IGF1R β IHC staining was membranous with no discernible cytoplasmic or nuclear labeling [Figure 2]. Immunostaining was variable and ranged from negative, low, to high staining. High IGF1R β expression was seen in 8/32 cases (25%). High staining did not correlate with a specific age group, gender, or tumor location [Table 1]. All the patients with high expression were dead at the end of follow-up. High expression was significantly associated with bad outcome ($P = 0.0048$). High IGF1R β staining also revealed a significant association with shorter OS [$P = 0.0398$, Figure 2]. Of these eight tumors, one was localized, three were metastatic, and four with regional extension. High staining also showed a trend, albeit statistically

insignificant, association with advanced tumors (regional extension + metastatic) ($P = 0.0534$).

Staining for p-S6K revealed distinct cytoplasmic labeling [Figure 2]. High p-S6K expression was noted in 22/32 (68.75%) cases and did not demonstrate any significant association with age, gender, or clinical stage. There was a trend but no statistically significant difference in OS between tumors with negative/low versus high expression on Kaplan–Meier curves ($P = 0.0934$). There was no correlation between the expressions of IGF1R β and S6K [Table 1].

Discussion

This study documents and confirms the importance of IGF1R β and S6K in EST growth and the results are congruent with few previous studies from *in vitro* cell lines and immunohistochemical studies that have revealed wide expression of IGF1R in EST and its downregulation after treatment with inhibitors.^[9-11] Our results, highlighting the worse prognosis associated with IGF1R expression, are in agreement with these findings which were the basis for using IGF1R inhibitors in several preclinical and clinical EST trials.^[9-11] However, other studies were contradictory and reported higher IGF1R mRNA and nuclear protein expression in association with better prognosis.^[12,13] The reason behind this discrepancy is not clear and may be related to methodical differences or to the presence of uncharted complexities in IGF1R pathway. Our study emphasizes only strong cytoplasmic staining of IGF1R expression as the predictor of bad prognosis.

In many clinical trials, EST treatment with various IGF1R inhibitors has resulted in resistance with less satisfactory results.^[6,14] Although more than 1 mechanism can explain this resistance as outlined earlier, cross-signaling and cross-talk with other signaling molecules may result in the activation of other downstream pathways, bypassing IGF1R inhibition and leading to the maintenance of tumor growth. Combined inhibitors of other signaling networks in a synergistic manner

Table 1: IGF1R expression in relation to clinicopathological data stratification, $n=32$

Clinicopathologic parameters	Data stratification	Number (%) with high expression	Total number of cases (%)	P-value
Age (years)	≤ 20	3 (9.4)	8 (25)	0.2479
	> 20	5 (15.6)	24 (75)	
Gender	Males	5 (15.6)	21 (65.6)	0.7078
	Females	3 (9.4)	11 (34.4)	
Patients' outcome	Alive	0 (6.7)	15 (46.9)	0.0048
	Dead	8 (18.8)	17 (53.1)	
Clinical stage	Localized	1 (6.7)	12 (37.5)	0.0534
	Advanced*	7 (18.8)	16 (50.0)	
	Unknown	0 (0)	4 (12.5)	
S6K expression	High	6 (18.8)	22 (68.75)	0.2565
	Negative/low	2 (6.7)	10	

*Distance metastasis and regional extension groups were added together as "advanced category" and statistically compared to the localized group

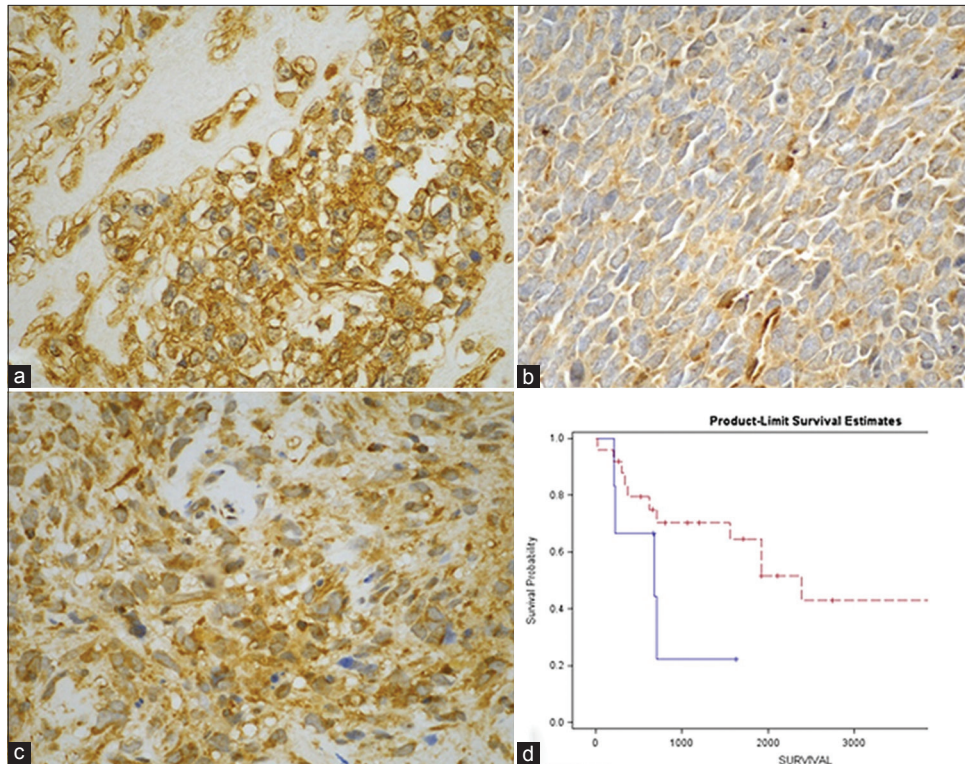


Figure 2: High staining with IGF1R β revealing strong diffuse membranous staining (a. $\times 200$). Faint positive staining that is weaker than endothelial staining is considered low (b. $\times 200$). High staining with p-S6K (c. $\times 200$) manifested as diffuse cytoplasmic labeling. (d) Kaplan-Meier survival curve revealed a significant difference in the overall survival between tumors expressing high (solid line) versus low/negative expressors (interrupted line -----), logrank $P = 0.0398$

have proven to be a more logical approach to overcome chemoresistance.^[15,16]

In this regard, we examined the expression of p-S6K in EST and highlighted a trend association with worse survival indicating a potential marker of poor prognosis in EST. Activation of S6K is part of the intracellular signaling cascade of IGF1R and plays a role in MAPK signal transduction. A previous study has revealed that S6K phosphorylation is linked to AKT apoptosis pathway and is decreased significantly with silencing or inhibition of the AKT substrate, PRAS40, which is another target of insulin action.^[17] S6K has been found to play a role in resistance to IGF1R inhibitor therapy in other tumors.^[18] In neuroblastoma cell lines, the activation of S6K was associated with decreased responsiveness to IGF1R inhibition.^[19] On the other hand, inhibition of S6K increases the sensitivity of colon carcinoma cells to IGF1R inhibitor.^[20] S6K is a central signaling molecule and its expression in EST makes it an attractive target for inhibitory therapy. Synergistic IGF1R/S6K inhibitors may help overcome EST resistance to IGF1R targeted therapy.

Conclusion

Although this study is limited by small number of patients and tumor samples, it helps to further elucidate the role of IGF1R β and S6K in Ewing's sarcoma signaling and

stresses the importance of only high IGF1R β staining as indicator of aggressive EST and potential clinical response to IGF1R inhibitory therapy. Further studies on p-S6K in EST, particularly with larger number of cases and statistical association studies, are necessary for better understanding of its role in EST targeted therapy.

Ethics Approval and Consent to Participate

The study was approved by the Institutional Review Board of University of South Florida. No individual patient consents were obtained due to the archival nature of the study.

Availability of Data and Material

The data used in this study are available and will be provided by the corresponding author on a reasonable request.

Competing Interest

The authors declare that there are no conflicts of interest.

Funding Statement

None.

Authors' Contributions

Dr. Elizabeth Gonzalez, a visiting student at Children's Mercy Hospital, analyzed the data. Dr. Marylin Bui provided patients materials included in the study. Dr. Ahmed finalized the manuscript.

Acknowledgment

The authors would like to acknowledge Joan Whiting of the Histology Laboratory of Children's Mercy Hospital for performing the immunohistochemical stains. The authors also would like to acknowledge and thank Dr. Ahmed Anwar for performing the statistical analysis; he could not be reached for authorship inclusion. This work has been presented in abstract form at the International Society for Pediatric Oncology annual meeting, Lyon, France 2019.

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